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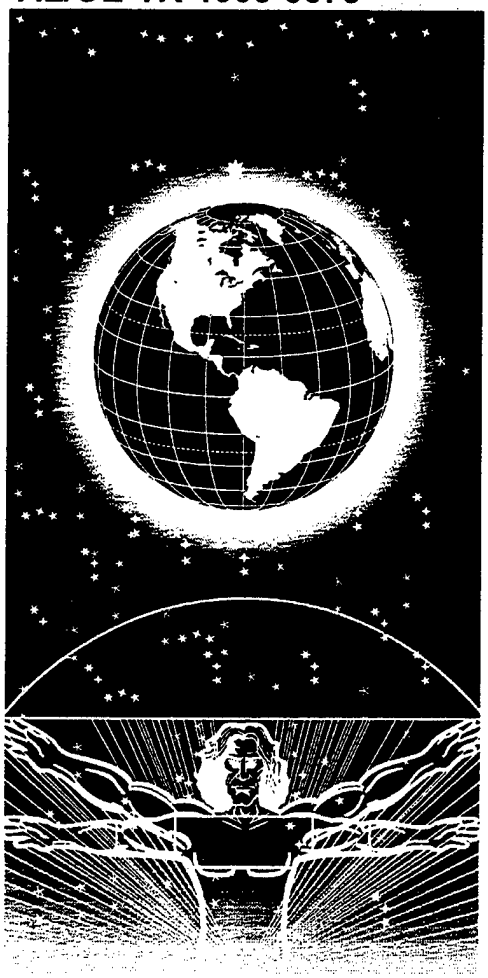

UNITED STATES AIR FORCE ARMSTRONG LABORATORY

USES OF EPR/SPIN LABELING AS A BIOMARKER

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FOR THE DIRECTOR

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Recently protein receptor changes has attracted interest for use as biomarkers in environmental toxicology. Insulin and transferrin which control cell growth were chosen in this study. There were two aims: first, to measure insulin and transferrin receptor binding using spin-labeled insulin and second; basic research on changes in receptor binding of human erythrocytes after exposure to radiation and chemicals likely to be encountered by military personnel in operational or combat scenarios. Insulin and transferrin were spin-labeled and incubated with red blood cells exposed to *y*-radiation or chemicals (ammonium dinitramide or mustard gas), the cells were centrifuged and receptor binding measured from the EPR spectra using a Bruker ESP300E and EMS104 spectrometer. Transferrin and insulin receptor binding was decreased with ammonium dinitramide. Transferrin receptors are only present in immature erythrocytes. Insulin receptor binding was decreased by *y*-irradiation and increased by mustard gas. Irradiated mustard gas did not increase insulin receptor binding in red blood cells. As most cells contain insulin receptors, insulin receptor binding is likely to be useful for risk assessment efforts and the spin label technique could be developed as a clinical tool not just for insulin but for other compounds with specific protein receptors on cell membranes.

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PREFACE

This is one of a series of technical reports generated from the experimental laboratory programs conducted in the Electron Paramagnetic Resonance (EPR) laboratory of the Armed Forces Radiobiology Research Institute with collaboration with the EPR laboratory at Occupational and Environmental Toxicology Division, Armstrong Laboratory. The collaborative research described in this report began in October 1993 and was completed in April 1996. This work has been presented at the Society of Armed Forces Medical Laboratory Sciences Conference, Washington DC in March 1996 and at the Conference on Advances in Toxicology and Applications to Risk Assessment. This research has been sponsored by the Armed Forces Research Institute work unit 04630 since 1991 and partially by the Air Force Office of Scientific Research, Independent Laboratory Investigator Research, 2300OT51 since 1995. Inquires on details in spin labeled protein methodology and mustard gas data should be directed to AFRRI. Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory, Toxicology Division.

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ABBREVIATIONS

ADN	**Ammonium dinitramide**
EPR	**electron paramagnetic resonance**
G	**Gray**
H-MG	**Half mustard gas**
rbc	**red blood cells**
nm	**nanometers**
m	**milli**
min	**minute**
M	**mole**
MNP	**2-methyl-2-nitroso-propane**
P	**probability**
r	**correlation coefficient**

SECTION 1

INTRODUCTION

Cell membranes can be categorized into different types according to their function and method of permeability. For example, liver cells have type I membranes. The typical type I membrane is about 5 nm thick and consists mainly of lipoid material mixed with some protein (Albert, 1985). Initial studies for this project were performed using liver cells (Carmichael et al., 1995 and Steel-Goodwin & Dean., 1995). Liver cells were excellent for our initial studies because as Type I membranes they represent the commonest type of membrane found in living organisms. However, liver cell receptor isolation is impractical for screening occupational and environmental risks in field studies. Based on our initial experiments, we assumed for this study that spin labels would be potential biomarkers of cell membrane damage.

The goal of this study was to express possible transitions of a protein receptor assay for occupational and environmental health studies. For transition studies we used human red blood cells which are classified as Type II membranes. Type II membranes are adapted to hasten the absorption of metabolites which could not be expected to penetrate a Type I membrane because of ionization or an excessively hydrophilic structure.

Red blood cells are the most abundant cells in the body and red blood cells contain many different protein receptors depending on their age and functional needs. For example, transferrin receptors which bind to the iron binding protein transferrin are

present in immature blood cells. However, when red cells mature and loose their nucleus, they loose the transferrin receptor. Insulin receptors are also present in red blood cells. Insulin receptors are present in almost every cell of the body. Unlike transferrin receptors, they are present in mature red blood cells. Many hazardous chemicals exert their deleterious effects through receptors or by dissolving into the lipid portions of the cell membrane and acting by altering the biophysical properties of those membranes. This study focused on the effects of ammonium dinitramide (ADN), and it transitioned to studying the effects of half mustard (H-MG) on the red blood cell membrane.

The Air Force is currently determining the suitability of using ADN, a high energy oxidizer, to improve solid rocket fuel performance. The Army is exploring neutralization and neutralization plus biodegeneration as an alternative to incineration of schedule I chemicals such as mustard gas.

This study had three aims:

1. To determine if ADN exerts a more predictive effect on the lipid or the protein portion of the red cell membrane when the effects of ADN on labeled probes which bind to the lipid portion of membranes and the protein receptors of transferrin in red cells are compared.
2. To compare the effects of ammonium dinitramide on the type of labeled probe which provides the best prediction in (1) to other deleterious effects such as cell irradiation and H-MG.
3. To see if the labeled probe used in (2) can demonstrate that radiation of H-MG neutralizes H-MG effects on red blood cell membranes.

The probes that were used were non-radioactive to decrease the use of hazardous materials in the laboratory. The probes were spin labels which are stable free radicals. Free radicals are paramagnetic and can be detected by electron paramagnetic resonance spectroscopy (EPR). Spin labels have been used for over twenty years to determine the physical structure of biological compounds. Spin labels are available commercially.

SECTION 2

METHODS

Chemicals

Insulin, transferrin and the spin label succinimidyl 2,2,5,5 tetra methyl-3-pyrolline-1-oxyl-3 were purchased from Calbiochem, Sigma and Molecular Probes respectively. Insulin and transferrin were spin labeled by a modification of the technique of Twining et al (1981). The concentration of the spin labeled proteins were determined by spectrophotometry. Other spin labels: 5-, 7-, 10-, 12-, 16, and 20-doxyl stearic acid were purchased from Sigma and Aldrich. Ammonium dinitramide (ADN) was obtained from SRI, CA., and half mustard (H-MG) was obtained from the USA.

EPR and EPR/Spin trapping

ADN and H-MG was irradiated in a ^{60}Co γ-ray source. The dose rate was 1 Gy per minute. The total dose was 25 Gy for ADN and 50 Gy for H-MG. The samples were immediately measured in a Bruker EMS 300E spectrometer. H-MG was spin trapped using the trap 2-methyl-2-nitroso-propane (MNP). MNP was purchased from Aldrich and was prepared as described previously (Carmichael & Reisz 1984).

EPR/Spin labeling

The experimental details for preparation of red blood cells and EPR/spin labeling have been described previously (Steel-Goodwin 1991 and Carmichael et al 1995). Briefly,

packed human red blood cells, prepared at the National Naval Medical Center Blood Bank, Bethesda MD or the clinical pathology laboratory OET, were exposed to ADN, 15 Gy γ-radiation from a ^{60}Co-gamma ray source, H-MG, or irradiated H-MG and then receptor binding was determined using a spin labeled probe. The amount of spin-label bound to the erythrocytes was measured using a Bruker EMS104 or a Bruker ESP300E spectrometer using techniques described previously (Steel-Goodwin & Hutchens 1995). Red cell numbers were measured using a clinical blood cell counter. The relative EPR signal intensity was measured and statistically monitored by analysis of variance.

Statistics: All data was analysed by regression analysis. Scatter plots were perfomed on spin labeled insulin.

SECTION 1

RESULTS & DISCUSSION

Figure 1 shows the spectra of solid ADN and H-MG following exposure to 25 Gy γ-irradiation. High frequency electromagnetic radiation, from UV via X-rays to γ-rays and also high energy particles such as neutrons, protons or α-particles (helium nuclei) if they have sufficient energy, have the capability to eject electrons from chemicals such as ADN and H-MG. In solid-state studies, EPR spectroscopy is the best detection method for studying radical intermediates following radiolysis (Rice-Evans et al 1991). Figure 1 shows a signal was detected by each chemical studied.

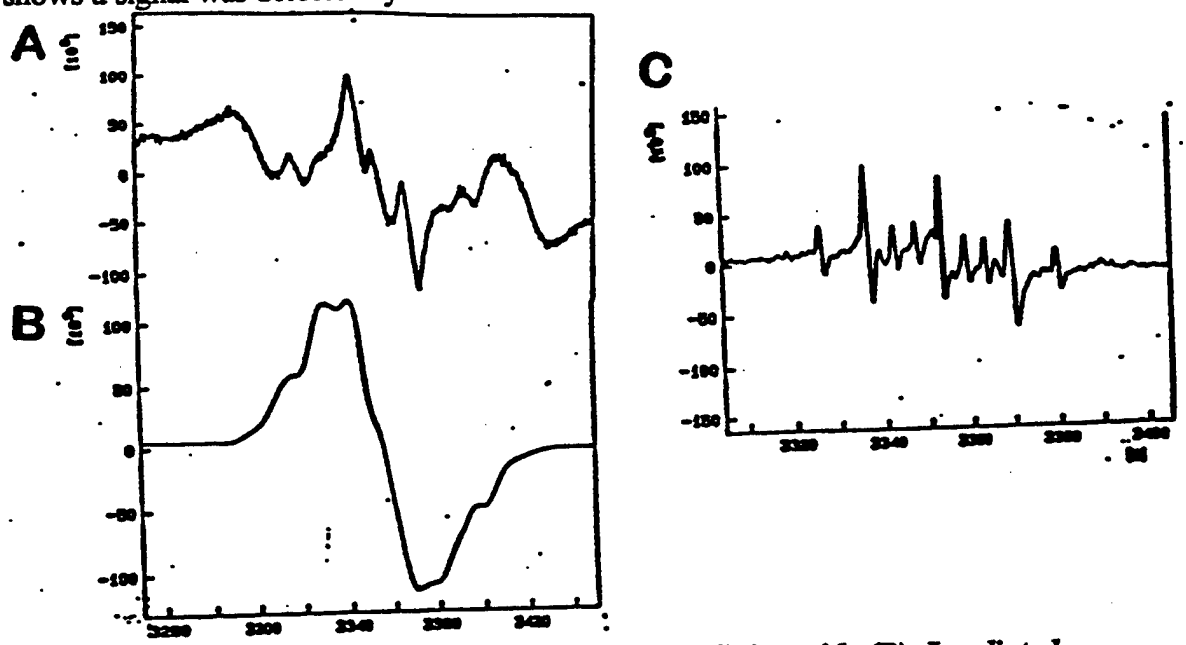
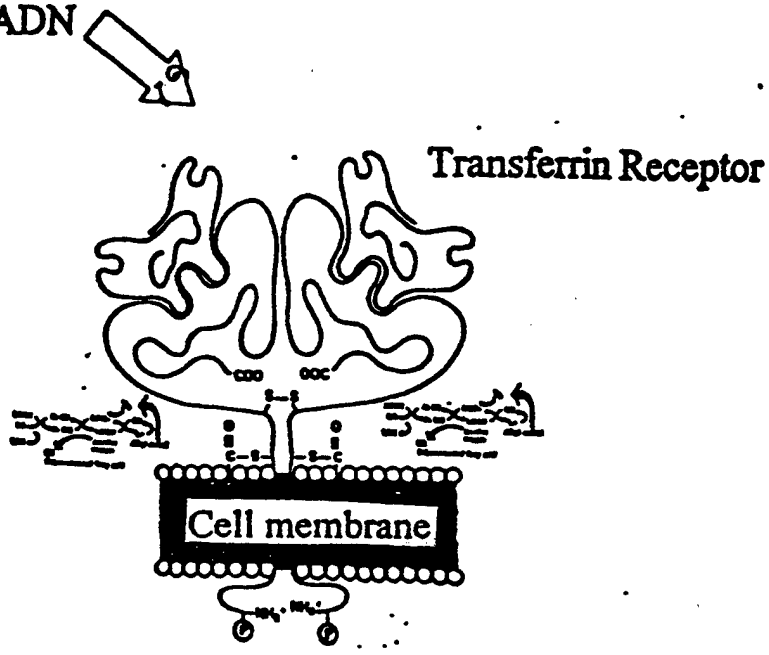


Figure 1 ***EPR spectra of (A) Irradiated ammonium dinitramide (B) Irradiated hydrogen mustard (C) Irradiated half mustard dissolved in MNP.***

The radicals generated by irradiation of ADN, Figure 1A have been described elsewhere (Steel-Goodwin et al 1995). This is the first demonstration of the radical

generated by H-MG, Figure1B. On dissolution in the spin trap, MNP, the spectrum shown in Figure 1C was obtained.

A



B

Label	r^2	F-Value	P-value
5-doxyl-	0.64	85.9	$4.05E^{-22}$
7-doxyl-	0.61	50.2	$1.54E^{-22}$
10-doxyl-	0.45	25.7	$2.91E^{-22}$
12-doxyl-	0.65	87.9	$2.03E^{-22}$
16-doxyl-	0.55	38.3	$2.61E^{-16}$
transferrin	0.92	288	$1.75E^{-37}$

Figure 2 ***(A) Cartoon of the transferrin receptor in a typical membrane. (B) Table of regression analysis of 5-, 7-,10-, 12-, and 16-doxyl stearic acid and spin labeled transferrin in red blood cells with and without exposure to ammonium dinitramide***

Figure 2A shows a cartoon of the structure of the transferrin receptor and Figure 2B is the table of the regression analysis results of the changes in EPR signal intensity of

each spin label tested with and without ADN. The regression data shows that the commercially available spin labels are not as specific as the transferrin spin label synthesized in-house. The doxyl spin labels enter the lipid portion of the membrane while the spin labeled transferrin is specific for the transferrin receptor. This experiment suggested that the spin labeled proteins synthesized provided more specific data than the non-developmental items already available commercially. Spin labeled stearic acid has been used to study membrane fluidity. Our assumption for the stearic acid studies was that ADN would affect membrane fluidity of red blood cells as well as their protein receptors because it is an oxidizer. ADN is a hydrophilic chemical and the regression analysis of the spin labeled stearic acid study, Figure 2B suggests ADN has a more selective effect on the proteins in cell membranes, such as transferrin receptors, than lipids. In these studies, transferrin receptor changes only measure the effects of ADN on immature red blood cells. The data results are given in Appendix A.

Both mature and immature red blood cells have insulin receptors. Figure 3 shows the curve of spin-labeled insulin binding in non-exposed red blood cells. The insulin binding curve was depressed following exposure to 1 mM ADN or γ-radiation. The dose of radiation, 15 Gy, is the dose approved by the Food and Drug Administration for preparation of red blood cells prior to transfusion into neonates or immunocompromised patients. At present there is no biomarker to ascertain if irradiated blood has any adverse effects on these patients. Irradiation of blood is necessary for patient survival to prevent transfusion associated graft versus host disease (Leitman, 1989).

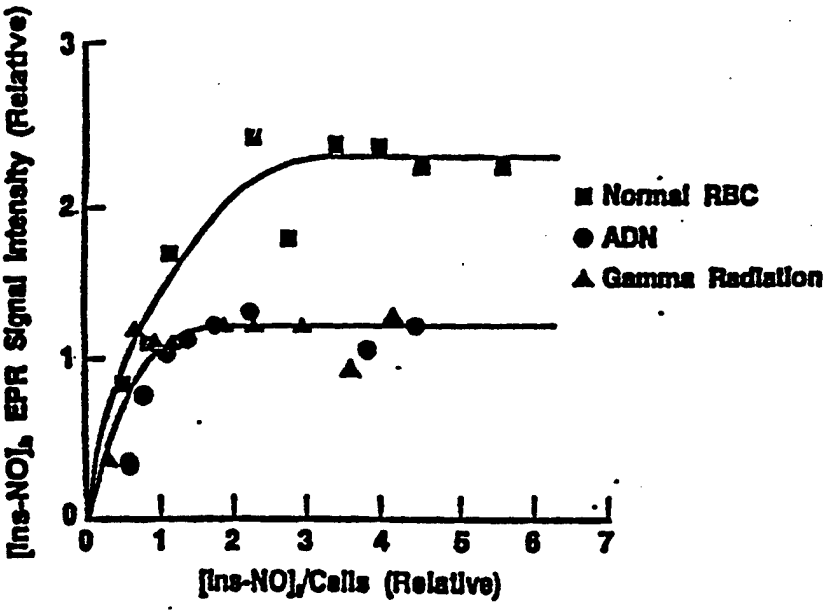


Figure 3 ***EPR signal intensity versus spin labeled insulin per cell in □ control red blood cells, • ammonium dinitramide treated red blood cells and Δ γ-irradiated red blood cells.***

The major function of red blood cells is to transport oxygen, a paramagnetic molecule. Oxygen transport is carried out in red cells by hemoglobin. Hemoglobin is carried bound in the red blood cells because it is toxic. Hemolysis occurs when the red blood cell membrane is damaged. Hemolysis of red cells can occur as a result of exposure of cells to radiation, physical damage, chemical damage or aging of the cell membrane. This spin label assay could be tailored to a clinical setting to determine if red blood cells are functioning correctly and are suitable for human transfusion.

ADN will cause hemolysis to red blood cells at concentrations of greater than 10 mM (results not shown). In these experiments, ADN had the same effect as irradiation on insulin receptor binding. This assay could be used as a biomonitor to determine risk of

ADN exposure in the work environment. The assay could even be tailored to mitigate risk of adverse effects on wild life exposed to ADN environmentally.

The mission boundaries of occupational and environmental toxicology are medical, safe work practices and environmental risk assessment related to health and safety of the environment and the worker. Health risks to workers can be much greater in a disposal or a clean-up environment than in a production site or laboratory. In production and the laboratory, the same work is performed in the same location and this work is repeated day after day, week after week and sometimes year after year. The health risks to the worker in production sites and laboratories are more likely to be well defined because of this repetition. However, at a disposal facility or in a clean-up site, workers could continually face new health hazards as the chemicals sent for disposal and the clean-up sites vary. This poses many challenges to occupational health risk assessment and also challenges to the type of assay chosen to determine a biological effect. A spin label assay could be used to determine changes in membranes. As the assay involves receptor effects even artificial membranes with purified receptors worn as a dosimeter or set at certain locations could be used. This could remove the requirement to draw blood and the receptor would be purified so binding should theoretically be more specific. As the technique of spin labeling requires the use of an external magnetic field and a means to detect the change in the magnetic field reading, this technique, in theory, could lend itself to remote monitoring or sensing.

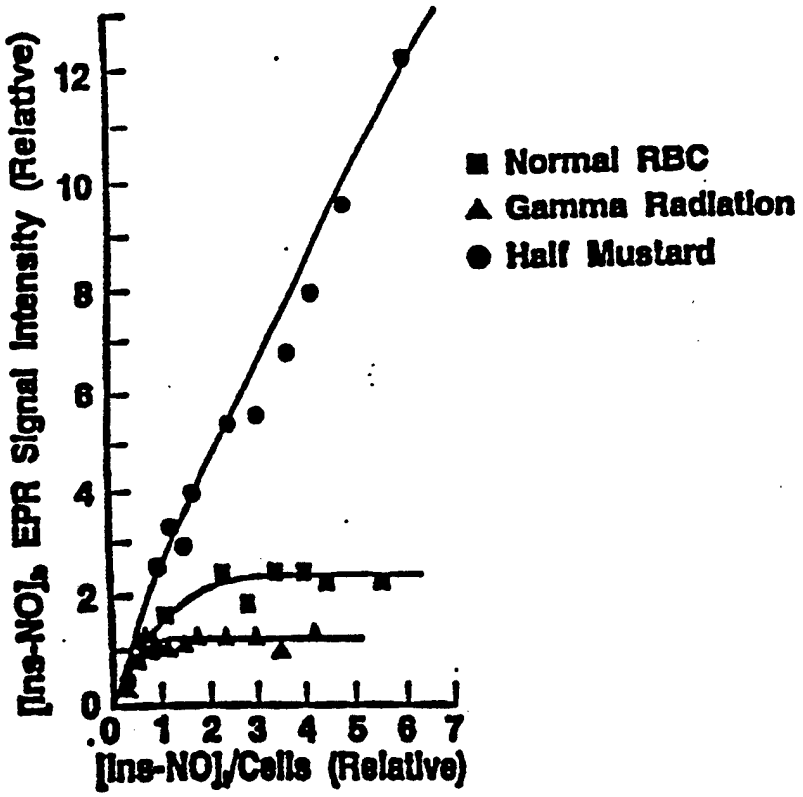


Figure 4 ***EPR signal intensity versus spin labeled insulin per cell in □ control red blood cells, • half-mustard treated red blood cells and Δ γ-irradiated red blood cells***

Figure 4 shows the erythrocyte binding response to spin labeled insulin after exposure to H-MG and γ- irradiation. In Figure 4, the relative EPR signal intensity was recorded with the concentration of spin-labeled insulin/cell. All data was plotted on the same curve. Binding of red blood cells to unexposed cells is curvilinear showing maximal binding to the spin labeled insulin. Equilibration studies between red blood cells and H-MG were not found in the literature but in vivo injections of ^{35}S-labeled MG suggest rapid diffusion and distribution of the radioactivity to all tissues were it becomes firmly fixed or bound to protein complexes (Boursnell et al 1946). In the present experiments

binding to insulin receptors in irradiated and non-irradiated red blood cells was increased with H-MG exposure but this excess binding was depressed when non-irradiated red blood cells were incubated with γ-irradiated H-MG. This experiment shows that H-MG does alter insulin binding to red blood cells. Also, there was no synergistic effect if red blood cells were exposed to γ-irradiation and then H-MG.

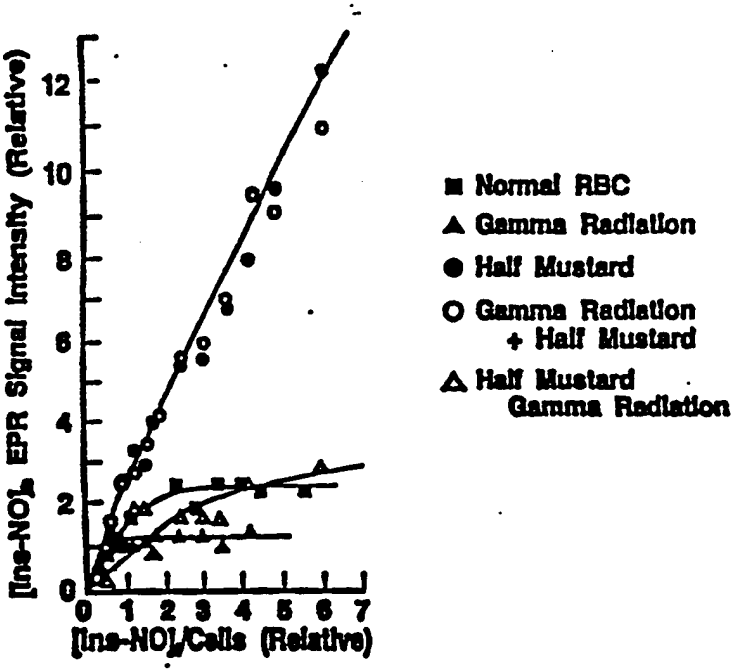


Figure 5 ***EPR signal intensity versus spin labeled insulin per cell in □ control red blood cells, • half-mustard treated red blood cells, ° γ-irradiated red blood cells treated with half mustard, Δ γ-irradiated red blood cells and red blood cells exposed to Δ γ-irradiated half-mustard.***

However, if the half mustard is irradiated and then exposed to red blood cells the biological effect of the H-MG on the red blood cells was drastically reduced, Figure 5. Therefore, the spin-labeled insulin did provide support to the theory that radiation will

mitigate the effects of H-MG. This assay could therefore potentially have uses as a biomonitor if radiation is used to destroy MG.

Defense of armed forces against the effects of chemical weapons is, nowadays, an essential component of defense in an international context. The requirements are numerous, complex, expensive but a necessity. Many Iranian victims of mustard gas attacks were treated in hospitals all over Europe in 1984-1986. The biological effects of mustard gas have been recently reviewed by Dacre and Goldman 1995 and Marrs et al., 1996. Foreign governments, including the U.S., recognizing the danger of unlimited and uncontrolled exportation of chemicals, have initiated means to control the export of chemicals which can be used for the production of chemical weapons. The mustard gases were chosen in this study because they are by far the easiest of chemical warfare agents to manufacture (Franse et al 1986) and there is an interest in finding ways of monitoring their destruction.

In conclusion, this poster shows the data obtained using spin-labeled probes to study red blood cell membrane damage. Spin-labeled probes are a more environmentally safe method to study receptor binding than radiolabelled assays, they are inexpensive to make, and the results can be read within 60 seconds even using the portable EMS104 spectrometer. Thus, there is potential to use the technique of spin labeling to study biological effects for occupational and environmental toxicology. The spin-label technique also has the potential to be adapted to other biomolecules suggesting it can be tailored to the needs of customers in the fields of environmental chemistry and medicine. The spin label assay could be used to investigate ways to reduce, eliminate, recycle and dispose of

hazardous chemicals, as an alternative tool to radiolabeled insulin (Gambir et al 1977., Ogunwole et al 1986., MacDonald et al 1991 and Grunberger 1993) in clinical medicine and for quality control of irradiated blood.

The technique of EPR/spin labeling:

- is a cost effective
- an alternative to radiolabels
- allows multilabeling
- is a method that provides extreme sensitivity of measurement
- reagents are easy to use
- system is environmentally safe
- is a proven technology.

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APPENDIX A

Chemical Codes for Appendix A

1 = 5-doxyl-; 2 = 7-doxyl-; 3 = 10-doxyl-; 4 = 12-doxyl-; 5 = 16-doxyl-;

6 = transferrin

chemical	LABEL	READING
1	20	1110.1
1	20	1111
1	20	1108.6
1	18	658.8
1	18	680.8
1	18	685.2
1	16	616.3
1	16	621.7
1	16	575.9
1	14	221.4
1	14	232
1	14	234.5
1	12	233
1	12	243.1
1	12	243.9
1	10	143.1
1	10	153
1	10	152.4
1	8	213.3
1	8	217.8
1	8	218.8
1	6	116.5
1	6	120
1	6	120.1
1	4	66.47
1	4	68.54
1	4	69.18
1	2	29.6
1	2	29.26
1	2	29.08
1	0	8.6
1	0	9.01
1	0	8.24
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2	20	418.2
2	20	420.5
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3	14	611.7
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3	2	244.2

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4	20	825.6
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4	14	406.2
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4	10	268.3
4	10	284.6
4	10	287.7
4	8	227.9
4	8	238.6
4	8	239.6
4	6	190
4	6	197.1
4	6	197
4	4	171
4	4	174.2
4	4	174
4	2	11.75
4	2	12.88
4	2	11.67
4	0	8.12
4	0	8.58
4	0	8.05
5	20	244.2
5	20	245.7
5	20	246
5	18	175.2
5	18	170.9
5	18	174.6
5	16	101.1
5	16	105.6
5	16	107.8
5	14	133.5
5	14	142
5	14	142.3
5	12	103.4
5	12	96.91
5	12	106.2
5	10	80.91
5	10	92.17

5	10	96.08
5	8	71.29
5	8	78.47
5	8	80.64
5	6	102.8
5	6	106.2
5	6	106.5
5	4	65.64
5	4	69.76
5	4	69.84
5	2	8.33
5	2	8.33
5	2	8.49
5	0	9.56
5	0	8.32
5	0	8.4
6	20	1006.5
6	20	1004.3
6	20	1002.3
6	18	867.9
6	18	880.4
6	18	879
6	16	725.2
6	16	732.4
6	16	730.8
6	14	716.5
6	14	721.7
6	14	718.9
6	12	598.7
6	12	601.6
6	12	605.8
6	10	371.6
6	10	373.7
6	10	370.2
6	8	373
6	8	350
6	8	357.2
6	6	209.6
6	6	210.5
6	6	217.1
6	4	89.29
6	4	88.21
6	4	85.16
6	2	47.85
6	2	44.95
6	2	46.4
6	0	34.36
6	0	31.64
6	0	30.86